

Expression of BRC Repeats in Breast Cancer Cells Disrupts the BRCA2-Rad51 Complex and Leads to Radiation Hypersensitivity and Loss of G₂/M Checkpoint Control*

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BRCA2 is a breast tumor suppressor with a potential function in the cellular response to DNA damage. BRCA2 binds to Rad51 through its BRC repeats. In support of the biological significance of this interaction, we found that the complex of BRCA2 and Rad51 in breast cancer MCF-7 cells was diminished upon conditional expression of a wild-type, but not a mutated, BRC4 repeat using the tetracycline-inducible system. Cells expressing a wild-type BRC4 repeat showed hypersensitivity to γ -irradiation, an inability to form Rad51 radiation-induced foci, and a failure of radiation-induced G₂/M, but not G₁/S, checkpoint control. These results strongly suggest that the interaction between BRCA2 and Rad51 mediated by BRC repeats is critical for the cellular response to DNA damage.

BRCA2 was cloned based on an analysis of mutations in families predisposed to breast cancer showing that a large percentage of the kindred had alterations within this locus (1, 2). The expression pattern of BRCA2 is remarkably similar to that of BRCA1 (3–5), with highest levels in the testis, thymus, and ovaries (5). At the cellular level, expression is regulated in a cell-cycle dependent manner and peak expression of BRCA2 mRNA is found in S phase (6). These results suggest BRCA2 may participate in regulating cell proliferation.

Recent studies indicate that BRCA2 is important for the cellular response to DNA damage. *Brca2*-null mouse embryos are nonviable at a very early stage of development and blastocysts derived from these embryos are very sensitive to γ -irradiation (7). Mouse embryonic fibroblasts predicted to express BRCA2 that is C-terminally truncated at amino acid 1492 also demonstrated sensitivity to DNA damaging agents, particularly methyl methanesulfonate and UV light (8). Furthermore, Capan-1, a human pancreatic cancer line, that expresses a 220-kDa C-terminally truncated BRCA2 protein, is hypersensitive to a panel of DNA damaging agents (9). Importantly, ectopic expression of wild-type, but not mutated, BRCA2 in Capan-1 cells restores resistance to treatment with MMS (10). These results provided convincing evidence that BRCA2 plays a critical role in the DNA repair process.

Interestingly, BRCA2 was shown to interact with Rad51 (7, 10–12), a key protein in DNA recombinational repair. Human *Rad51* encodes a 40-kDa protein with a structure related to the

Escherichia coli recombination protein RecA (13) and mediates homologous DNA pairing and strand exchange (14, 15). Similar to *mBrca2*, inactivation of mouse *Rad51* results in an embryonic lethal phenotype, indicating that Rad51 protein is essential for development (16, 17). Beyond serving as a DNA repair protein through its interactions with other Rad proteins including Rad52 and Rad54 (18), how Rad51 may participate in cell growth and development remains unclear.

While an association between BRCA2 and Rad51 is well documented, there is, nonetheless, some discrepancy concerning the regions of BRCA2 that bind to Rad51. It was reported that the C-terminal region of mouse *Brca2* binds to mouse Rad51 (7). By contrast, we and others have previously shown that the BRC repeats located in exon 11 (amino acid 1009–2083) of human BRCA2 bind to Rad51 (10, 12). There are eight repeats in BRCA2 designated as BRC1 to BRC8 (Fig. 1A) (19, 20). BRC1, BRC2, BRC3, BRC4, BRC7, and BRC8 are highly conserved and bind to Rad51, whereas BRC5 and BRC6 are less well conserved and do not bind to Rad51 (10, 12). Whether the interaction between BRCA2 and Rad51 has biological significance remains completely unknown. In an effort to investigate this issue, we have used the tetracycline binary gene control system for conditional expression of a BRC4 repeat in breast cancer MCF-7 cells. In this communication, we have found that upon expression of a wild-type, but not a mutated, BRC4 repeat, the interaction between BRCA2 and Rad51 was reduced. Cells expressing a wild-type BRC4 repeat showed hypersensitivity to γ -irradiation, an inability to form radiation-induced Rad51 nuclear foci, and a failure of radiation-induced G₂/M checkpoint control. These results strongly suggest that the BRC repeats of BRCA2 are important for mediating the cellular response to DNA damage.

EXPERIMENTAL PROCEDURES

Mutagenesis and Selection for BRC Mutants—The 39-amino acid BRC1 repeat (residue 1003–1042 of BRCA2) (2) in pBSK (Stratagene, La Jolla, CA) was randomly mutagenized by a biased-pool PCR¹ method (21) using T3 and T7 primers. Deoxynucleotide triphosphates used in PCR reaction were 10 mM dGTP, dCTP, dTTP, and 2 mM dATP. The resulting PCR fragments were digested with *Nco*I and *Xho*I and cloned into pAS1 vector (22) to generate a BRC1 mutant library (pAS1/BRC1-ML). These plasmid DNAs were transformed along with pGAD-Rad51 into the yeast Mav203 strain (MAT α , leu2–3, 112, trp1–901, his3d200, ade2–101, gal4d, gal80d, SPAL10:URA3, GAL1::lacZ, HIS3UAS GAL1::HIS3@LYS2, can1R, cyh2R) and selected for 5-fluoroorotic acid-resistant colonies (23). The recovered pAS1/BRC DNAs were further sequenced to determine the mutations. β -Galactosidase activities (U) was measured using chlorophenyl-red- β -D-galactopyranoside as substrate for interactions in the yeast two-hybrid system as described (22).

Isolation of Cell Clones with Inducible Expression of the BRC Re-

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¹ The abbreviations used are: PCR, polymerase chain reaction; GFP, green fluorescence protein; DAPI, 4',6-diamidino-2-phenylindole; BrdUrd, bromodeoxyuridine; Tet, tetracycline.

TABLE I
Identification of mutations in the first and fourth BRC repeats of BRCA2 that disrupt binding to Rad51

A randomly mutagenized pool of cDNAs encoding BRC1 repeats (amino acid 1003–1042) was cloned into the pAS1 vector and co-transformed along with pGAD-Rad51 into Mav203 cells. Four clones with DNA inserts that showed no detectable β -galactosidase activity in a yeast two-hybrid assay were isolated. Mutations with amino acid changes resulted from single nucleotide changes. Rad51 binding activity using the BRC4 in pAS1 vector was also tested in the assay. Note that this repeat has 3-fold higher activity compared with the BRC1 repeat. The T to A mutation identified above in the BRC1 repeat that abrogates Rad51 binding, and a familial mutation identified in BRC4 (G1529R) was introduced into the BRC4 and tested in the yeast-two hybrid assay. Both of the mutations significantly reduced, but the G to R mutation did not completely eliminate, Rad51 binding in this assay.

BRC consensus	I I L D FXTASGKX X SXXXLKKXXX X V V F E	Relative β -galactosidase activity
BRC1	FRTASNKEIKLSEHNIKKSKMFFKD	7
BRC1-M1	A	<1
BRC1-M2	G	<1
BRC1-M3	S	<1
BRC1-M4	RG	<1
BRC4	FHTASGKKVKIAKESLDKVNLFDE	22
BRC4-M5	A	<1
BRC4-M6	R	4
vector		1

peat—To generate cell clones that express a GFP-BRC4 fusion protein, we employed the tetracycline-inducible expression system controlled by a *tet*-responsive promoter (24). A pUHD10-3-based plasmid was used to construct pUHD10-3/GFP-BRC plasmids that will express chimeric proteins containing GFP with a nuclear localization signal and a myc epitope fused to either a wild-type BRC4 or a mutated BRC4-M5. These two plasmids were separately co-transfected into MCF-7 cells with the second plasmid, pCHTV, which contains a hygromycin resistance gene and a cytomegalovirus-controlled tetracycline repressor-VP16 fusion transcription unit. Cell clones resistant to hygromycin were subsequently isolated and several of them were shown to express the wild-type or mutant GFP-BRC4 upon removal of tetracycline. The expression of GFP-BRC4 was further confirmed by immunoprecipitation with α -myc 9E10 monoclonal antibody (25) and immunoblotting analysis with a monoclonal α -GFP antibody (CLONTECH, Palo Alto, CA). Two stable lines of MCF-7 cells, WT-8 and MT-11, that conditionally express wild-type BRC4 and BRC4-M5 mutant, respectively, were established.

Immunoprecipitations and Western Blotting—Immunoprecipitations were performed as described previously (10). Co-immunoprecipitations were performed similarly but with lysis buffer containing 180 mM NaCl. Antibodies specific for BRCA2 (10), human Rad51, Ab-1 (Oncogene Science, Cambridge, MA) and myc 1-9E10 monoclonal antibody (25) were used for the immunoblotting analysis according to standard procedure (10).

Immunostaining Rad51 Foci—Procedures for immunostaining were adapted from Zhong *et al.* (26). Briefly, cells in Dulbecco's modified Eagle's medium with or without tetracycline (1 μ g/ml) grown on coverslips to 60–70% confluence were irradiated with 12 Gy using a Mark I, model 68A irradiator. After 6 h, cells were fixed in 4% formaldehyde with 0.1% Triton X-100. α -Rad51 antibody (Ab-1) diluted 1:1000 in 10% goat serum was added onto the cells and then visualized with goat anti-rabbit antibody conjugated to Texas Red. Cells were further stained with 4',6-diamidino-2-phenylindole (DAPI) and mounted in Permafluor (Lipshaw-Immunonon, Pittsburgh, PA). Rad51 foci-positive cells were counted and recorded using a standard fluorescence microscope.

Clonogenic Survival Assay—Cells (WT-8 and MT-11) were seeded in identical plates at 5000 cells/plate in medium with tetracycline (1 μ g/ml). Expression of the wild-type or mutant GFP-BRC4 repeat was induced by removing tetracycline 24 h after seeding. Twenty four hours after induction of GFP-BRC4 expression, cells were then γ -irradiated with 3 Gy. After incubation for 14 days, cells were fixed and stained with 2% methylene blue in 50% of ethanol for colony counting. Averages and standard deviations were determined from eight plates. Statistical analyses were performed with the programs InStat and InPlot (Graph-Pad Inc., San Diego, CA).

Cell Cycle Checkpoint Analysis—The G_1/S checkpoint was determined according to the procedures described (27). Briefly, cells in logarithmic growth were mock-exposed or γ -irradiated (12 Gy). After 24 h, cells were labeled with 10 μ M BrdUrd for 4 h and fixed for BrdUrd staining using a Cell Proliferation Kit (Amersham Pharmacia Biotech). BrdUrd-positive cells were quantified, and expressed as a fraction of the

total cells. For the G_2/M checkpoint, cells were irradiated to 3 Gy, fixed with 4% paraformaldehyde at indicated time and stained with DAPI for counting mitotic cells in prophase, metaphase, anaphase, and telophase (28). Alternately, cells were irradiated with 4–16 Gy and processed for analysis of mitotic cells after 1 h.

RESULTS AND DISCUSSION

To systematically address the biological consequence of the interaction between BRCA2 and Rad51, amino acid residues of the first BRC repeat, BRC1, that are critical for Rad51-binding were first examined. BRC1 was subjected to biased PCR mutagenesis (21), and the mutated cDNAs were translationally fused to the GAL4 DNA-binding domain in the yeast vector, pAS1 (22), to generate a library of 2×10^6 individual clones referred to as pAS/BRC1-ML. A reverse two-hybrid screen with negative selection was used to isolate clones that fail to bind Rad51 as described previously (23). Several mutations in BRC1 were identified that significantly reduced Rad51 binding in a yeast two-hybrid assay (Table I). BRC1-M1 is a mutation that changes a conserved threonine residue to alanine. BRC1-M2 and -M3 are changes in nonconsensus amino acids, and BRC1-M4 carries a double mutation at the two C-terminal BRC1 residues, the last residue of which is conserved. Interestingly, a familial mutation, G1529R, has been previously found in BRC4 (Breast Cancer Information Core). Specific Rad51 binding activity by BRC4 was also tested and found to be approximately three times stronger than BRC1 (Table I). Two BRC4 mutations, BRC4-M5, an analogous mutation to BRC1-M1, in which the conserved threonine at the third position is changed to an alanine, and BRC4-M6, which contains the G1529R mutation, were constructed and found to have reduced Rad51-binding (Table I). These results suggest that, despite their sequence conservation, the ability of BRC repeats to bind Rad51 varies, and is dependent on specific residues.

To determine the functional importance of the interactions between the BRC repeats of BRCA2 and Rad51, two stable lines of MCF-7 cells, WT-8 and MT-11, that conditionally express wild-type BRC4 and mutant BRC4-M5, respectively, were established (Fig. 1B). Tetracycline-responsive expression of the GFP-BRC4 fusion proteins in these two lines was clearly demonstrated by immunoprecipitation with α -myc antibodies and immunoblotting with α -GFP or α -Rad51 antibodies (Fig. 1C, top panel compare lanes 2 and 4 with 1 and 3). Rad51 is detected in the immunoprecipitates of wild-type, but not GFP-BRC4-M5 (Fig. 1C, compare lanes 4 with 2), indicating that the

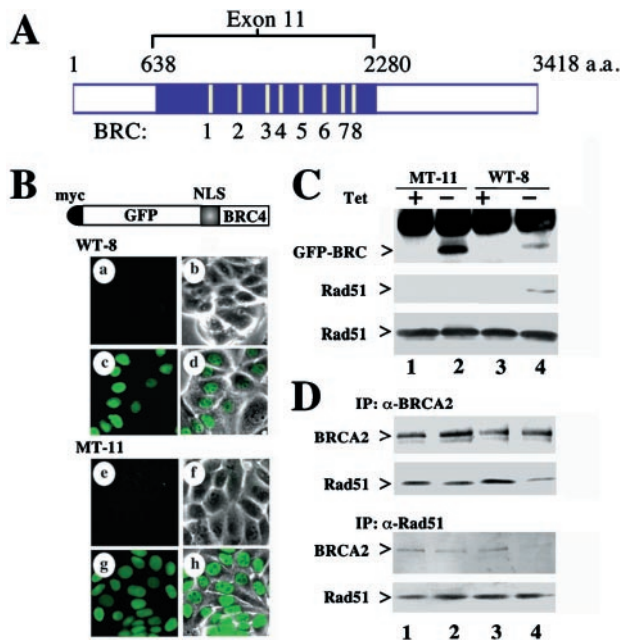


FIG. 1. Conditional expression of wild-type and mutated GFP-BRC4 fusions in MCF7 cells. *A*, diagram of BRCA2 showing exon 11 and the eight BRC repeats. *B*, the *top panel* is a schematic drawing illustrating the GFP-BRC4 fusion containing a BRC4 cDNA fragment translationally fused to a myc epitope-GFP-nuclear localization signal cassette in a modified pUHD10-3 plasmid, pUHD10-3/GFP-BRC. The *lower panel* shows cell clones expressing the wild-type GFP-BRC4 (WT-8) (*panels a-d*) and mutated GFP-BRC4-M5 (MT-11) (*panels e-h*) were visualized by GFP autofluorescence (*panels a, c, e, and g*) after incubation in the presence (*panels a, b, e, and f*) or absence (*panels c, d, g, and h*) of tetracycline (*Tet*). Fluorescence overlaid with phase-contrast images (*panels b, d, f, and h*) show nuclear localization of these fusion proteins. *C*, co-immunoprecipitation of GFP-BRC4 with Rad51 in cells. Cells expressing wild-type (WT-8) or mutated GFP-BRC4 (MT-11) were immunoprecipitated with α -myc antibody (*top two panels*), immunoblotted with either α -GFP to detect the GFP-BRC4 fusions or α -Rad51 (indicated in the *left margin*). Immunoprecipitation and Western blotting with α -Rad51 antibody (*bottom panel*) determined the relative levels of endogenous Rad51. *D*, expression of wild-type GFP-BRC4 in WT-8 cells reduces the complex formation between BRCA2 and Rad51. Thirty-six hours after induction of GFP-BRC4 expression, cell lysates were co-immunoprecipitated with either α -BRCA2 (*top panel*) or α -Rad51 antibody (*bottom panel*). The resulting immune complexes were analyzed by immunoblot analysis with either α -BRCA2 or α -Rad51 antibody, indicated on the *left margin*.

GFP fusion with wild-type BRC4 binds to Rad51 in cells.

Importantly, expression of wild-type, but not the BRC4-M5 mutant, significantly reduced BRCA2 in the Rad51 immunoprecipitates and, in the reciprocal experiment, reduced Rad51 in the BRCA2 immunoprecipitates (Fig. 1*D*, compare *lane 4* with *lane 2*). These data strongly suggest that conditional expression of a wild-type, but not a mutated, BRC4 repeat effectively disrupts the interaction between BRCA2 and Rad51.

The important role of Rad51 in recombinational DNA double-strand break repair (13) suggests that disruption of the interaction between BRCA2 and Rad51 may have an adverse effect on the ability of cells to respond to DNA damage. To test this possibility, both WT-8 and MT-11 cells cultured either with or without tetracycline, were mock-exposed or γ -irradiated (3 Gy), and cell survival was determined by clonogenic assay. Induction of the expression of a wild-type BRC4 repeat (WT-8, -Tet) significantly reduced cell survival rate when compared with the uninduced (WT-8, +Tet) or to either the induced (MT-11, -Tet) or uninduced (MT-11, +Tet) mutant BRC4 ($p < 0.0001$) (Table II).

To further explore DNA damage response phenotypes of these two cell lines, the appearance of radiation-induced

TABLE II

Clonogenicity of WT-8 and MT-11 cells after γ -irradiation

Actively growing WT-8 and MT-11 cells (5000) were either induced (-Tet) or uninduced (+Tet) to express GFP-BRC4. About 24 h later, cells were either mock-exposed or γ -irradiated (3 Gy) and cultured for 14 days. Survival rates by colony formation (>50 cells/colony) were determined by counting the number of colonies per plate. Averages and S.D. were calculated from eight plates. Survival rates were calculated by dividing the number of colonies in the mock-exposed control by the number from exposed cells. Note that the expression of wild-type, but not mutated BRC4 repeat, significantly reduced cell survival in this assay ($p < 0.0001$).

	0 Gy	3 Gy	Survival rate, mean \pm S.D. ($n = 8$)
WT-8 (+Tet)	405 \pm 14	189 \pm 12	46.8 \pm 7.9%
WT-8 (-Tet)	358 \pm 17	72 \pm 2	20.1 \pm 2.0%
MT-11 (+Tet)	371 \pm 10	173 \pm 10	46.8 \pm 8.0%
MT-11 (-Tet)	379 \pm 15	163 \pm 7	43.2 \pm 5.2%

Rad51-containing foci was examined. Under uninduced conditions (+Tet), both clonal lines formed Rad51 foci after γ -irradiation (Fig. 2*A*). However, WT-8 cells induced to express a wild-type GFP-BRC4 repeat exhibited a reduction in the appearance of Rad51 foci compared with MT-11 cells induced to express the GFP-BRC4-M5 mutated repeat (Fig. 2*A* for representative field and Fig. 2*B* for quantification). These data suggest that the interaction between BRCA2 and Rad51 is crucial for the formation of Rad51 repair foci and, furthermore, that exogenous expression of BRC repeats can interfere with this activity.

Increased sensitivity to ionizing radiation may result from defects in the DNA repair machinery or in the molecules essential for cell cycle checkpoint control. When normal mouse embryo fibroblasts are exposed to γ -irradiation, their transit through the cell cycle is arrested at either one of two points (27, 29). The G_1/S checkpoint, dependent on p53 and p21 (29–32), prevents the replication of damaged DNA. The G_2/M checkpoint prevents segregation of damaged chromosomes (33). To test for a potential role of BRCA2-Rad51 interactions in DNA damage-induced cell cycle checkpoint control, cells expressing the GFP-BRC4 repeat were assayed for G_1/S and G_2/M checkpoint integrity in response to γ -irradiation. As shown in Fig. 3*A*, WT-8 and MT-11 cells, under all conditions, demonstrated nearly identical numbers of BrdUrd-incorporated cells, indicating that the expression of the BRC repeats did not significantly impair G_1/S checkpoint control in response to γ -irradiation.

In contrast, when cells were assayed for mitotic figures at variable times after γ -irradiation, the number of cells in mitosis was not significantly decreased in WT-8 cells induced to express a wild-type BRC4 repeat (Fig. 3*B*, *panel a*). However, MT-11 cells induced to express a GFP-BRC4-M5 mutated repeat, as well as uninduced cells, demonstrated significant reductions in mitotic figures (Fig. 3*B*, *panel a*). In a parallel experiment, cells were irradiated with 4–16 Gy. In the population induced to express a wild-type BRC4, the percentage of mitotic cells was significantly higher than that of cells either uninduced or induced to express mutant GFP-BRC4-M5 (Fig. 3*B*, *panel b*). These results suggest that cellular expression of a wild-type BRC4 repeat interferes with the radiation-induced G_2/M checkpoint.

It was reported that mouse embryo fibroblasts, with a genotype *Brc2^{tm1Cam}* and predicted to express a C-terminally truncated BRCA2 at amino acid 1492, have intact cell cycle checkpoint responses (8). This truncated BRCA2 protein possesses the first three BRC repeats (34). The abrogation of embryonic lethality by *Brc2^{tm1Cam}* (8) compared with other truncating mutations that delete exon 11 of all BRC repeats (7) strongly suggests that the remaining 3 BRC repeats in the

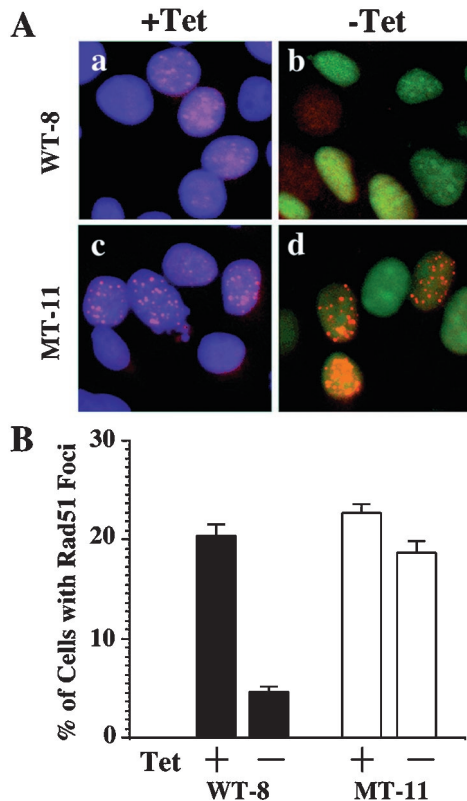


FIG. 2. Radiation-induced Rad51 foci were diminished in cells expressing wild-type GFP-BRC4. A, WT-8 and MT-11 cells (indicated in the left margin) were cultured in the presence (+) or absence (-) of tetracycline (*Tet*) for 48 h and then exposed to 12-Gy γ -radiation. Irradiated cells after 6 h were immunostained with α -Rad51 and Texas Red-conjugated secondary antibody. Expression of wild-type and mutant GFP-BRC4 was visualized by GFP autofluorescence. Panels *a* and *c* show the radiation-induced Rad51 foci overlaid with DAPI in the uninduced cells (+*Tet*). Panels *b* and *d* show the radiation-induced Rad51 foci overlaid with GFP autofluorescence in the induced cells (-*Tet*). Note the radiation-induced Rad51 foci were diminished in the wild-type GFP-BRC4 expressing cells (panel *b* compared with panel *d*). B, images containing 250 cells were captured by computer, and the number of cells containing at least 10 foci were recorded and plotted as a percentage of total cells. The plots were generated from two independent experiments.

Brca2^{tm1Cam} truncated protein are partially functional. The functional importance of the BRC repeats is supported by our results demonstrating that the expression of GFP-BRC4 repeat in cells results in hypersensitivity to radiation and a failure in G_2/M checkpoint control. The increased radiosensitivity in cells expressing the BRC4 repeat indicates that the complex of BRCA2 and Rad51 is important for the mechanics of the repair process. This notion is compatible with the known role of Rad51 in recombination repair. In this capacity, BRCA2 may facilitate Rad51 function in strand exchange by modulating formation of the Rad51-DNA nucleoprotein filament and/or pairing and strand-exchange steps of DNA double strand break repair (13).

The failure of radiation-induced G_2/M checkpoint control in cells expressing BRC4 repeat indicates that BRCA2 may have a role in this task. It is possible that the formation of the BRCA2 and Rad51 complex could be important for radiation-induced G_2/M checkpoint control. However, the BRCA2-Rad51 complex is formed independently of DNA damage (10–12). Other G_2/M checkpoint proteins may be required to participate in the BRCA2-Rad51 complex. Interestingly, mouse embryo fibroblasts expressing exon 11-deleted *Brca1* also exhibit defects in radiation-induced G_2/M checkpoint control (28) and BRCA2 apparently interacts with BRCA1 (35). It is possible, therefore, that the BRCA2-Rad51 complex may interact with

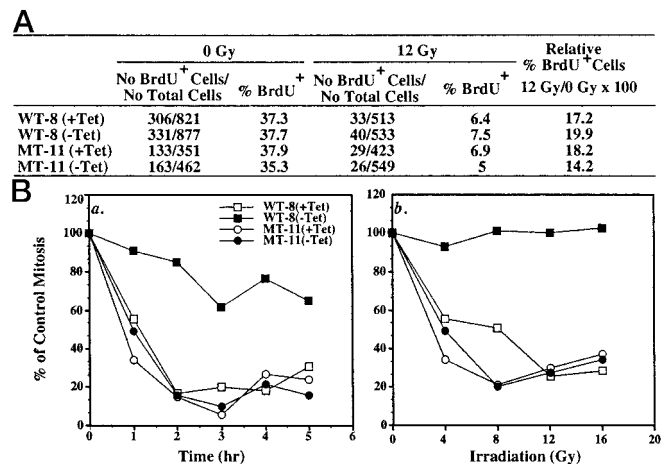


FIG. 3. Cells expressing GFP-BRC4 show intact G_1/S and defective G_2/M checkpoints. A, G_1/S checkpoint in γ -irradiated cells. WT-8 and MT-11 cells were incubated with (+) or without (-) tetracycline for 24 h and then mock-exposed (0 Gy) or γ -irradiated (12 Gy). Cells were labeled 24 h later with BrdUrd for 4 h and immunostained with α -BrdUrd antibody. The percentage of BrdUrd-positive cells was quantified and expressed as a fraction of the total number of cells. Note that the relative percentage of BrdUrd-positive cells decreases significantly in cells expressing both wild-type (WT-8) and mutated (MT-11) GFP-BRC4 following radiation, indicative of intact G_1/S checkpoint in these cells. B, defective G_2/M checkpoint in cells expressing wild-type GFP-BRC4. Mitotic cells were counted at 1–5 h after exposure to 3 Gy γ -radiation (panel *a*) or at 1 h after exposure of 4–16 Gy (panel *b*). Mitotic indices of mock-exposed cells were used as controls. Over 2000 cells were counted at each exposure and time point.

BRCA1 to establish G_2/M checkpoint control. Alternatively, the BRC repeats may mediate separate interactions with cell cycle checkpoint proteins when induced by DNA damage signaling.

Regardless of which possibility is operative in cells, the data presented here support and extend the model originally proposed (10), that BRC repeat interactions with Rad51 are important for the cellular DNA damage response. The observation that *mRad51^{-/-}* mouse embryos are not viable and ES cells with the same genotype cannot survive (16, 17), coupled with the observation that *Brca2^{tm1Cam}* cells are viable but radiation-sensitive (8), suggests that Rad51, through interactions with BRCA2, may have functions in addition to DNA repair. Like, BRCA1, it appears that BRCA2 functions in a pathway that bifurcates into one that is important for repair of genetic lesions and another important for restraining cell division until repair is complete. It is possible that each of the BRC repeats, or certain group of the repeats, may have separate and distinct functions within each pathway. Nevertheless, the fact that expression of one BRC repeat can disrupt Rad51-BRCA2 interactions, interfere with the G_2/M checkpoint, and can make cells radiation-sensitive suggests that it could be used to radiosensitize and/or chemosensitize resistant tumors.

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Checkpoint Control**

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